

**CO-IMMOBILIZED
ENZYMES AND COFACTORS**

by

Stina Gestrelius

1976



**DEPARTMENT
OF
BIOCHEMISTRY**

AVDELNINGEN FÖR BIOKEMI



**CHEMICAL CENTER
LUND INSTITUTE OF TECHNOLOGY — UNIVERSITY OF LUND
LUND-SWEDEN**



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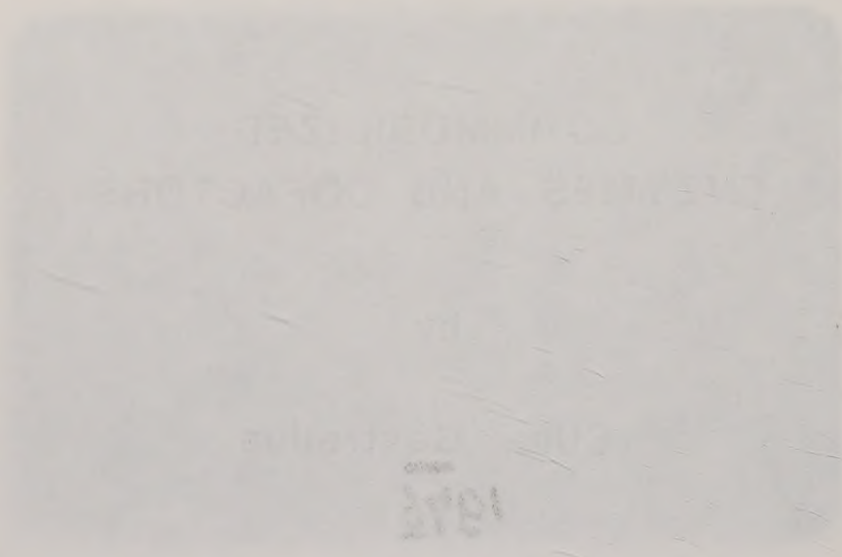
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av

Stina Gestrelus

Civ. ing., Mlm

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TILL MIN KÄRA DOTTER,
Pernilla

"En lärd i stubb-det är ett rön-
satirens udd ej undanslipper,
och vitterheten hos vårt kön
bör höra blott till våra nipper."

A.M. Lenngren 1797

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Proximity of enzymes and/or cofactors can be maintained by their co-immobilization on the same matrix.

For multi-enzyme systems such co-immobilization permits exploitation of diffusion-dependent microenvironmental effects, e.g. local enrichment of reaction products, giving high overall efficiency and new possibilities for fast regulation of enzyme activities.

Co-immobilization also results in good interaction between a cofactor(effector)-dependent enzyme and its cofactor (effector). Model systems can be run continuously when the cofactor is regenerated *in situ* by the two-substrate method. Under certain conditions cofactor regeneration was instead accomplished by acriflavin that had been co-immobilized with the enzyme.

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This thesis is based on the following publications, which are referred to by Roman numerals in the text.

- I. On the Regulation of the Activity of Immobilized Enzymes:
Microenvironmental Effects of Enzyme-Generated pH Changes
S. Gestrelus, B. Mattiasson and K. Mosbach (1973) Eur.J.Biochem.
36, 89-96.
- II. Studies on pH-Activity Profiles of an Immobilized Two-Enzyme System
S. Gestrelus, B. Mattiasson and K. Mosbach (1972) Biochim.
Biophys.Acta 276 339-343.
- III. Immobilization of Glycogen Phosphorylase b in Its Active Conformation
on Matrices Substituted with an AMP-Analogue
K. Mosbach and S. Gestrelus (1974) FEBS Lett. 42, 200-204.
- IV. Preparation of an Alcohol-Dehydrogenase-NAD(H)-Sepharese Complex
Showing No Requirement of Soluble Coenzyme for Its Activity
S. Gestrelus, M.-O. Månsson and K. Mosbach (1975) Eur.J. Biochem.
57, 529-535.
- V. Continuous Regeneration of NAD(P)^+ by Flavins Covalently Bound to
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M.-O. Månsson, B. Mattiasson, S. Gestrelus and K. Mosbach (1976)
Biotech.Bioeng. 18, 1145-1159.

INTRODUCTION

Immobilized enzymes can be described as enzymes that are in some way bound to a matrix or by other means retained in a reactor, and are thus possible to reuse. Immobilization can be accomplished, for example, by attachment to a solid support, by precipitation or by entrapment within a matrix lattice. A great number of methods have been developed during the last ten years for coupling enzymes, most often via covalent bonds, to all kinds of solid supports.

There are several reasons for using the immobilization technique. For biochemists it is a means of creating a model of natural enzyme-membrane systems, in the hope of obtaining better understanding of the enzyme behaviour at the cellular level. For engineers it is a way of introducing enzymes as heterogeneous catalysts in large scale continuous industrial processes. Immobilized enzyme processing is then comparable to other catalysis methods using soluble enzymes in batch procedures, free or immobilized whole cells, or non-enzymatic catalysts with generally very low specificity.

Immobilized enzymes have also proved to be useful in analytical devices such as enzyme electrodes, enzyme thermistors, reagent strips etc., since they are easy to handle and can be used repeatedly. Their use in the medical sphere is under development for enzyme replacement therapy, artificial organs etc. but has to overcome the problems, for example, of administration and toxicity.

There are of course different requirements of immobilized enzymes used in biological research and for technical applications, but there is also a common need for basic knowledge of the influence of such matrix properties as, for example, charge, hydrophobicity, porosity and stability, since these can alter the characteristics of the enzymes. Model studies have been valuable for investigation of these effects, one parameter at a time.

Many enzymes and other useful biomolecules have been tried for immobilization, and several immobilized enzymes are already being used in industrial processes or as analytical tools. Some examples are given in Table 1. However, these enzymes all belong to what has been called the first generation of immobilized enzymes, and they are single enzymes without any cofactor requirements. The coming generations, not yet in practical use, will include cofactor-dependent enzymes as well as

multi-enzyme systems.

The present model studies have been concerned with such enzyme-(cofactor-) systems, and some advantages regarding efficiency, stability and cofactor-utilization for co-immobilized enzymes and cofactors are discussed.

TABLE 1. Examples of immobilized enzymes that are being used in industrial processes and analytical devices. (From Skinner, 1975)

IMMOBILIZED ENZYME	APPLICATION
Glucose isomerase	Production of high-fructose syrup
Aminoacylase	Production of L-amino acids
α -amylase	Degradation of starch to dextrins
Glucoamylase	Production of glucose
α -galactosidase	Hydrolysis of raffinose
Penicillin amidase	Production of 6-aminopenicillanic acid
Glucose oxidase	Analysis of glucose
Urease	Analysis of urea
β -lactamase	Analysis of penicillins

METHODOLOGICAL ASPECTS OF CO-IMMOBILIZATION

Co-immobilization is a technique that can be described as the simultaneous immobilization of several enzymes, an enzyme and a cofactor, or any two or more biomolecules that will benefit by being adjacent on the same matrix.

The alternative methods available for systems of several enzymes (cofactors, etc.) are

- 1) sequential immobilization of the molecules to the same matrix
- 2) immobilization on separate matrices
- 3) use in free solution.

Another related possibility is to bind two different molecules covalently together, thereby creating a soluble bifunctional system. Such a two-enzyme system was developed by Mattiasson et al. (1974 a), and an enzyme-cofactor system by Venn et al. (1976). Enzyme-effector systems have also been described, for example by Hulla and Fasold (1972), and enzyme-antigen compounds are used in an enzyme linked immunosorbent assay (Sharpé et al., 1976).

Crosslinking of two enzymes followed by immobilization of the complex to a matrix has also been used (Hultin, 1974).

A matrix bearing two enzymes has been called a dual immobilized catalyst in contrast to a mixed immobilized catalyst, which implies the use of separate matrices (Fig. 1). A comparison between dual and mixed immobilized catalysts is given in the discussion (p.30).

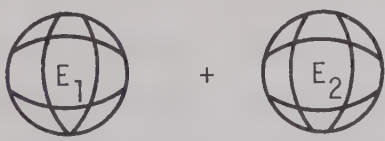
	MIXED CATALYSTS	DUAL CATALYSTS
SOLUBLE ENZYMES	$E_1 + E_2$	$E_1-----E_2$
IMMOBILIZED ENZYMES		

Fig. 1. Schematic representation of soluble and immobilized two-enzyme systems

1. Immobilization methods and matrix effects.

For two or more enzymes being co-immobilized, most of the classical matrices and coupling methods can be used since they involve binding to functional groups (carboxy-, amino-, phenolic-, mercapto-groups) common in almost all proteins. Entrapment within membranes or gel lattices is equally possible for these large molecules.

For cofactors, the immobilization procedure can be more laborious, since the cofactor must very often first be derivatized, that is a functional group suitable for binding must first be introduced in the molecule. Several different AMP(ADP, ATP)-analogues and NAD(P)-analogues have been synthesized, especially for use in affinity chromatography. The cofactor analogues used in the present study (III-V), N^6 -(6-aminoethyl)AMP and N^6 -(6-aminoethyl)carbamoylmethyl -NAD, were prepared according to Guilford et al. (1972) and Lindberg et al. (1973) respectively. Since cofactors are small molecules, the entrapment technique cannot usually be applied, due to problems of leakage, unless the cofactor is bound to a soluble polymer of a high enough molecular weight.

While enzymes and other polymer molecules are bound via multipoint attachment, cofactors are usually coupled via only one bond, thus making it very important that this is stable. Many of the classical coupling methods have recently been shown to give unstable bonds (Tesser et al., 1974, Wilchek et al., 1975). Rapid leakage of flavins coupled by the CNBr-, glutaraldehyde- or carbodiimide-methods was also found in the present study (V), and binding to epoxy-activated Sepharose was the only immobilization procedure tried which gave high enough operational stability for acriflavin. Ligand leakage has already been recognized as a problem in affinity chromatography and must be taken into consideration at the construction of enzyme-cofactor reactors.

The chemical and physical nature of the matrix has a large influence on the properties of co-immobilized enzymes, since it affects enzyme kinetics as well as stability and choice of reactor.

Immobilization of an enzyme very often results in a decreased specific activity due to denaturation effects and conformational changes (cf. p. 10) or steric limitations on the penetration of high molecular weight substrates (Goldstein, 1976).

An apparently changed kinetic behaviour can be caused by local matrix-microenvironments that differ from the milieu in the surrounding bulk solution, where pH and concentrations of substrates, products, inhibitors etc. are determined (Katchalsky et al., 1971).

Charged and hydrophobic matrices create microenvironments with altered concentrations of charged or hydrophobic molecules respectively. This results in, for example, apparently displaced pH optima and apparently changed Michaelis constants for charged (or hydrophobic) substrates (Goldstein et al., 1964, Johansson and Mosbach, 1974), but the effects can be reduced with high salt concentrations (or hydrophobic solvents).

The impeded diffusion of substrates and products in the vicinity of the matrix can also create a special microenvironment and affect immobilized enzymes. This is most evident for large matrix particles and thick membranes bearing high enzyme activity, especially under slow mixing conditions (for a stirred tank reactor) or low flow rates (for a packed column). Such a diffusion resistance causes increased Michaelis constants, less substrate inhibition but enhanced product inhibition for an immobilized enzyme compared to a soluble one (Engasser and Horvath, 1974 a, b, c).

If protons are produced or removed as a result of enzyme activity, the microenvironmental pH will become different from the bulk pH, leading to displaced and distorted pH optima (Goldman et al., 1968); effects that can, however, be reduced with high buffer capacities (Engasser and Horvath, 1974 d).

In the present study relatively uncharged matrices were used for co-immobilization of enzymes, but high salt concentrations were also used to eliminate any charges that had been introduced with the proteins. Displaced pH optima and increased Michaelis constants were found for enzymes such as glucose oxidase, trypsin and urease that had been entrapped in 25 % polyacrylamide beads (I), while enzymes bound to the smaller agarose beads (Sepharose 4 B) with their larger pore radii, seemed to be less influenced by impeded diffusion (II).

Good stability against mechanical strain and microbial attack is another very important factor in the choice of matrix. Among the materials used in the present study, porous glass has a good resistance against microorganisms, like all inorganic carriers, but it

is easily fragmented in a stirred batch reactor. Polyacrylamide beads have a relatively good mechanical stability as well as microbial resistance, and due to their small pores also exclude any exoenzymes, that might be produced by microorganisms (Koch-Schmidt, 1976). However, agarose beads were used in most of the model studies using stirred batch technique. Although this matrix is not very stable against microbial attack, it was considered a good choice since it has a good mechanical stability and can be loaded with high concentrations of enzymes and cofactors.

2. Determination of enzyme and cofactor content.

Most classical methods for protein determination do not discriminate between different proteins and are thus not useful for preparations containing more than one enzyme. Enzymes containing metal ions or prosthetic groups can however be determined independently. Thus, a combined method was used in this study (I). Since one enzyme, trypsin, was in very large excess, the protein content as determined by amino acid analysis was taken as that of the trypsin. The second enzyme, glucose oxidase with FAD as prosthetic group, was determined from the FAD content, which could be analysed fluorimetrically after acid hydrolysis.

Nucleotide-containing cofactors such as AMP and NAD(H) were determined by phosphate analysis or from absorbance spectra of gel samples suspended in glycerol (III, IV). The latter technique was also used for the determination of bound acriflavin (V).

3. Assay of enzyme activities.

Several modified techniques suitable for assaying the activities of immobilized enzymes have recently been reviewed (Mattiasson and Mosbach, 1976).

In the present study simultaneous measurement of the activities of three co-entrapped enzymes could be carried out (I). Two activities were followed spectrophotometrically by use of repetitive scanning and a flow cuvette, while the third activity was measured titrimetrically

in the reaction vessel.

In multi-step enzyme reactions the activity of the last enzyme in the sequence is determined by measuring the overall reaction product, while the activities of the preceding enzymes must be determined separately, for example by measuring via a product that is not used up in subsequent reactions.

However, very often the potential maximum activity of each enzyme is of greater interest, for example for preparation of comparable soluble systems (cf. p. 11), and this is determined in an excess of substrate at the pH optimum of the respective enzyme.

For dehydrogenases co-immobilized with their cofactor, NAD(P), the classical assay procedures cannot be used, for obvious reasons since they depend on determination of free cofactors (oxidized or reduced). In such cases either cofactor-regeneration or main product formation can be followed, continuously (IV) or intermittently (V).

4. Ratio and distribution of co-immobilized enzymes and cofactors.

Although all the enzymes are bound through the same kind of functional groups on immobilization, the different effective concentrations of reactive groups, as well as differences in size and charge of the enzymes, make the preparation of matrices with different predetermined ratios between enzymes a trial and error process. Entrapment within membranes or in very large pores makes the control of protein ratios easier.

However, the most interesting ratio is generally not the one between different amounts of proteins but the one between the resulting enzyme activities, which is of importance for the kinetic behaviour of the system (II).

A typical problem was co-entrapment of trypsin, glucose oxidase and hexokinase (I), where the last enzyme proved to be extremely labile in the presence of free trypsin and under the polymerization conditions. An active preparation could only be obtained after inhibiting trypsin with benzamide, and adding glucose-6-phosphate and ATP (product and substrate) to hexokinase together with cysteine, which protects sulfhydryl groups by dilution. Moreover, it was necessary to mix the hexokinase and trypsin solutions only 30 seconds before initiation of the polymerization.

The presence of cofactors, substrates or inhibitors that can protect the sensitive sites of enzymes during immobilization was also shown to give higher specific activities of Sepharose-bound phosphorylase b (III) and alcohol dehydrogenase (IV). The allosteric behaviour of enzymes such as phosphorylase b and phosphofructokinase can be influenced as well (unpublished results), but in only one case has the presence of native substrate during immobilization been reported to freeze an enzyme in the most active conformation (Uy et al., 1976).

The distribution of large amounts of enzymes in matrices and on matrix surfaces has for a long time been assumed to be uniform, and several authors have recently confirmed this assumption for at least some matrices and coupling conditions (Barboutin and Thomas, 1974, Lasch et al., 1975). However, the spatial distribution of two or more enzymes after covalent coupling to a matrix is probably less uniform, due to different reactivities of the proteins with the matrix and different molecular weights, causing divergent steric and diffusional hindrance.

On co-immobilization of an enzyme with a cofactor the dissociation constant (K_{diss}) between the two will affect their distribution in relation to each other (IV). If the K_{diss} is low (high affinity), the enzyme and cofactor can be bound to the matrix as a binary complex. This was shown in the present study when alcohol dehydrogenase (ADH) and NADH were co-immobilized on Sepharose. The very efficient interaction obtained between ADH and NADH when both were bound to the matrix even at low total concentrations and with equimolar ADH/NADH ratios was interpreted as evidence that the complex formation prior to binding had been retained on the matrix, at least in part. This interaction decreased to almost zero when a comparable enzyme-cofactor-matrix was prepared under conditions not permitting complex formation, although the ratios between bound enzyme and cofactor, and the concentrations, were almost identical (cf. p 25). Thus it is not the local concentrations of cofactor and enzyme per se that are decisive, but rather the orientation of the cofactor in the vicinity of the enzyme, thereby permitting interaction.

When, on the other hand, the dissociation constant is higher, as between phosphorylase b and its effector AMP, the molecules are bound

independently to a greater degree, and a large excess of nucleotide relative to enzyme must prevail in the matrix to give an efficient interaction. Thus the AMP/phosphorylase ratio used was 1700:1.

5. Preparation of comparable soluble systems.

There is no obvious way of making fair comparisons between immobilized and soluble enzyme systems. Generally, equivalent activities in identical assay volumes are compared (Mosbach and Mattiasson, 1970) and this method was used in the present study.

The soluble system is then made up by mixing the same separate activities as was determined for the co-immobilized system. However, the soluble molecules are dispersed in the total assay volume, while the immobilized enzyme is kept within the much smaller matrix phase. This may have implications for the stability since many enzymes are more easily denatured in dilute solution, an effect that was noticed for both horse liver alcohol dehydrogenase and glycogen phosphorylase b.

For cofactor-containing systems, the comparable soluble systems are prepared with an identical amount of cofactor. It is obvious however, that only a fraction of the cofactor on the matrix is sterically available for interaction with co-immobilized enzymes. In these cases, the soluble systems appear at least as effective as the immobilized, but if the assay volume is increased the immobilized system will become by far the most efficient, since it is not influenced by dilution.

CO-IMMOBILIZED ENZYMES

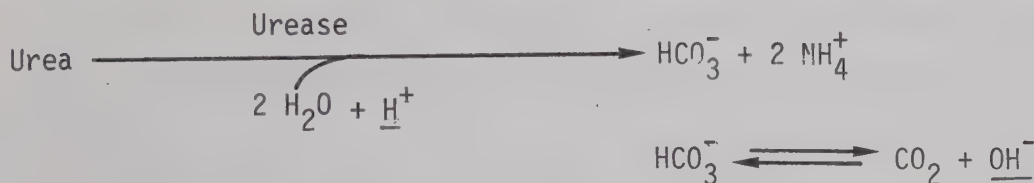
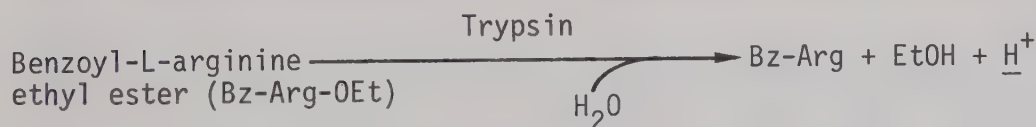
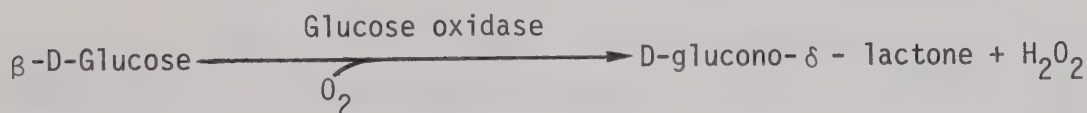
Co-immobilization of enzymes is undertaken to utilize two features, proximity and microenvironment. The proximity concept simply indicates the shorter statistical mean distance between two co-immobilized enzymes compared to that in solution, while the microenvironment concept (cf. p. 7) is a function of the nature of the matrix and of the impeded diffusion of substrates and products through the Nernst diffusion layer and through the pores within the matrix.

While proximity is a static consequence of co-immobilization, some micro-environmental effects can be exploited or quenched. One of these effects, the enrichment of reaction products in the microenvironment, can have profound influence on the activities of co-immobilized enzymes. This has been shown in model studies where one product is an activator or inhibitor for another enzyme (I, Lecoq et al., 1975) or when the product of one enzyme reaction is the substrate for another in a multi-step enzyme system (Mosbach and Mattiasson, 1970, II).

1. Regulation by enzyme-generated pH changes in co-immobilized enzyme systems (I).

Glucose oxidase activity was studied after co-entrapping the enzyme with trypsin or urease in polyacrylamide beads to observe the influence of changes in microenvironmental pH caused by the activities of trypsin or urease.

The enzymes perform the following reactions:



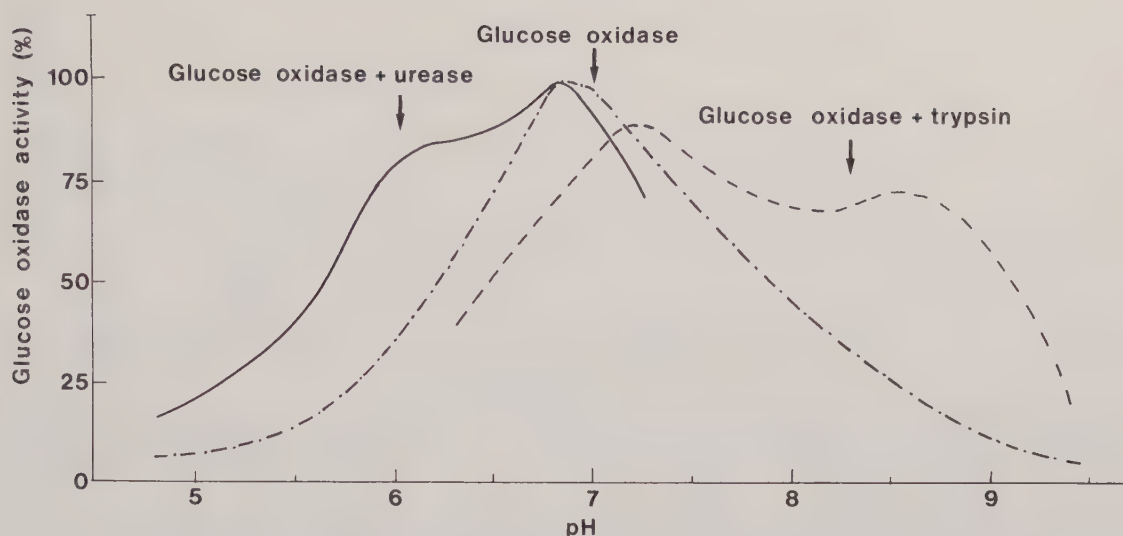


Fig. 2. Glucose oxidase activity influenced by simultaneous activity of co-entrapped trypsin or urease at different constant pH values of the bulk solution.

The two-enzyme matrices were assayed in weak buffers and the bulk pH was kept constant by titrating with base (during trypsin activity) or acid (during urease activity). The resulting apparent pH profiles of glucose oxidase are depicted in Fig. 2.

The displaced and broadened pH optima can intuitively be explained as follows, assuming glucose oxidase and trypsin are co-entrapped.

At high pH values trypsin activity is high (apparent $pH_{opt} = 9.5$), leading to an acidified microenvironment, with pH closer to the optimum of glucose oxidase, and thus increased glucose oxidase activity. When the bulk pH is kept constant at lower values, trypsin activity is lower (for example 50 % at pH 7.5) but the optimum of glucose oxidase is approached. The result is a broad pH curve for glucose oxidase, a profile that is almost insensitive to bulk pH between 6.5 and 9.

An analogous discussion can be applied to the results from co-entrapped glucose oxidase and urease (the latter has apparent $pH_{opt} = 5.8$).

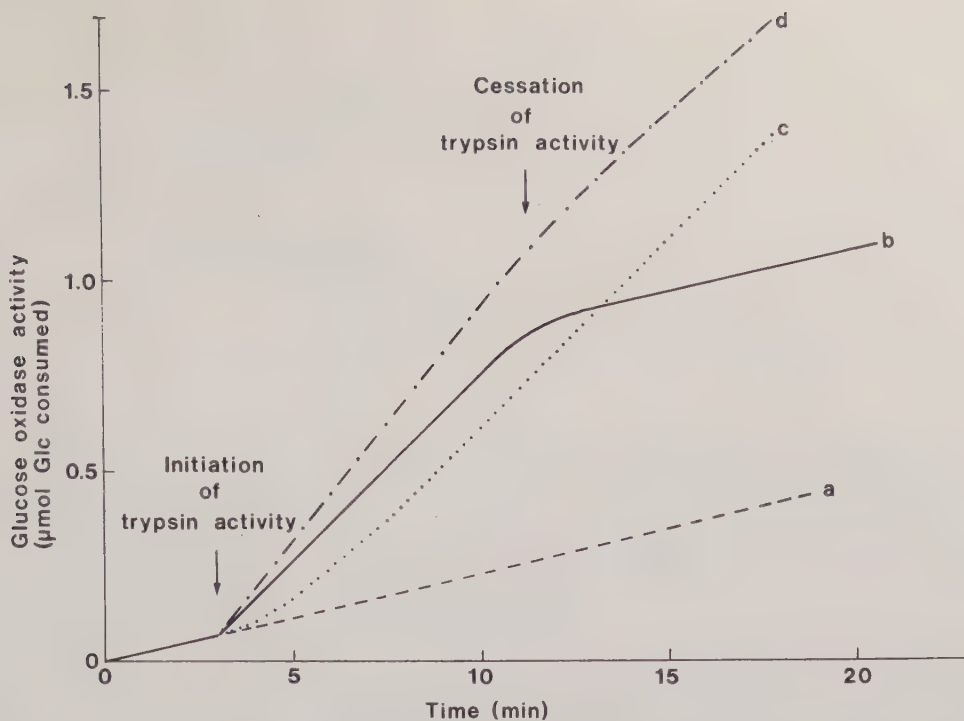
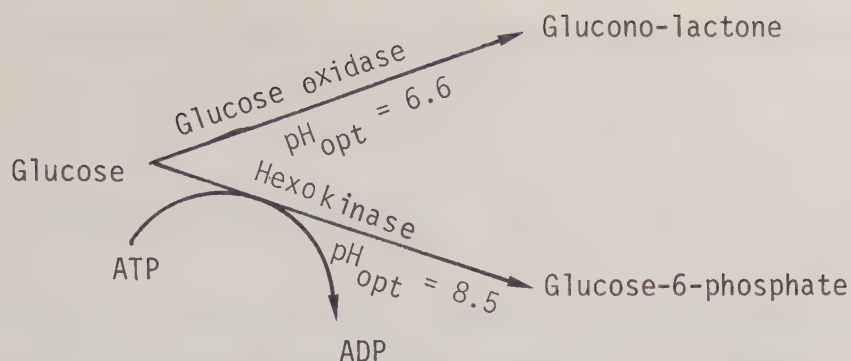


Fig. 3. Glucose oxidase activity before, during and after simultaneous trypsin activity.

- a. Either co-entrapped enzymes in strong buffer or free enzymes or separately immobilized enzymes in weak buffer if bulk pH was kept at 8.6 by titration.
- b. Co-entrapped enzymes in weak buffer; bulk pH was kept at 8.6 by titration.
- c. Free enzyme systems in weak buffer; bulk pH decreased from 8.6 to 7.0 due to trypsin activity.
- d. Co-entrapped enzymes in weak buffer; bulk pH decreased as in c.

In Fig. 3 a comparison is made between co-entrapped enzymes and separately entrapped or free enzymes. When bulk pH is kept constant by titration there is no influence at all of trypsin activity on separately entrapped or free glucose oxidase (3 a), but in the absence of titration, an effect appears after a lag phase since the bulk pH is gradually acidified (3 c). This behaviour is clearly different from the sharp changes in activity of the co-entrapped glucose oxidase (3 b)

The fast response of co-immobilized enzyme systems was also used to simulate a possible regulating mechanism of microenvironmentally generated protons. A three-enzyme system comprising glucose oxidase, hexokinase and trypsin, all co-entrapped in polyacrylamide gel, was prepared. Glucose oxidase and hexokinase both compete for the same substrate, glucose:



At pH 8.5, 13 % of the total glucose consumed was converted to glucose-6-phosphate by hexokinase. However, if trypsin activity was initiated this portion decreased to zero, since the internal pH became closer to the optimum of glucose oxidase (Fig. 4).

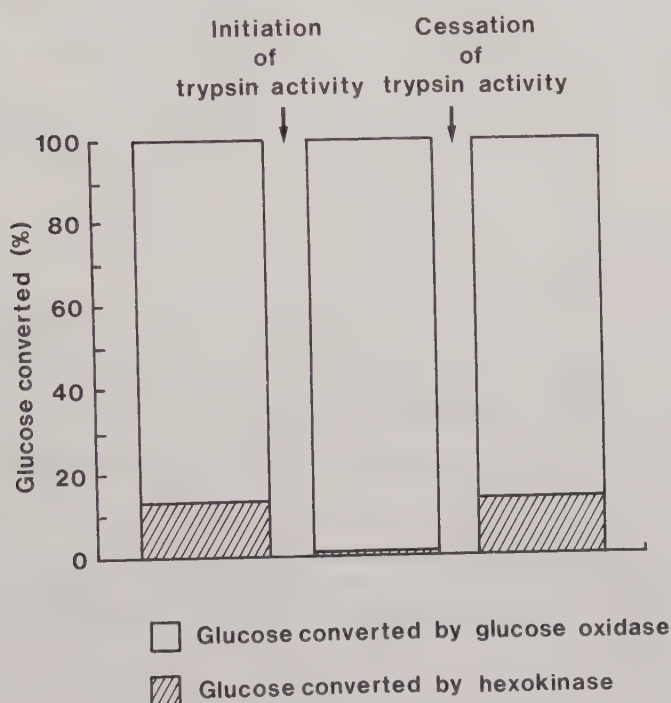


Fig. 4. Results of a typical experiment on the "regulatory" effect of trypsin activity on hexokinase and glucose oxidase activities towards 0.65 mM glucose at pH 8.5. The glucose converting enzyme activities are expressed as a percentage of the total amount of converted glucose per minute (mean value 21.5 nmol/min). The three columns show the steady state situations before, during and after trypsin activity.

The obvious advantages of exploiting these effects of enzyme generated microenvironmental pH changes are thus:

- high glucose oxidase activity in a broad pH interval (insensitive to bulk pH)
- possibilities of specific changes (increase/decrease) in glucose oxidase activity by a microenvironmentally generated effector

- regulative possibilities for controlling the direction of conversion for a particular metabolite (such as glucose).

2. Multi-step enzyme systems

Multi-step enzyme systems, systems of several enzymes working in sequence, exhibit perhaps the greatest advantages of co-immobilization, since the overall reaction can be completed within the microenvironment without substantial release of intermediates into the bulk solution (Fig. 5). This was first shown by Mosbach and Mattiasson (1970) for a two-enzyme system, the overall reaction being faster (without lag) during the initial period of reaction when the enzymes were co-immobilized, compared to when they were soluble or immobilized in separate matrices (Fig. 6). The results were ascribed to the faster build-up of the concentration of intermediates in co-immobilized systems, and the effect has been explained theoretically by Goldman and Katchalsky (1971).

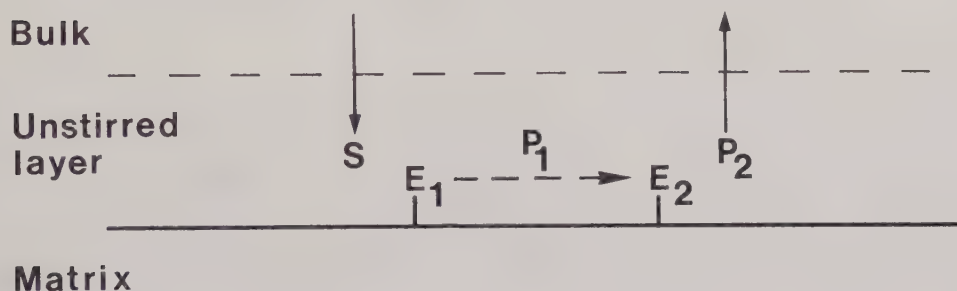


Fig. 5. Schematic representation of the diffusion of substrates and products in a co-immobilized two-enzyme system.

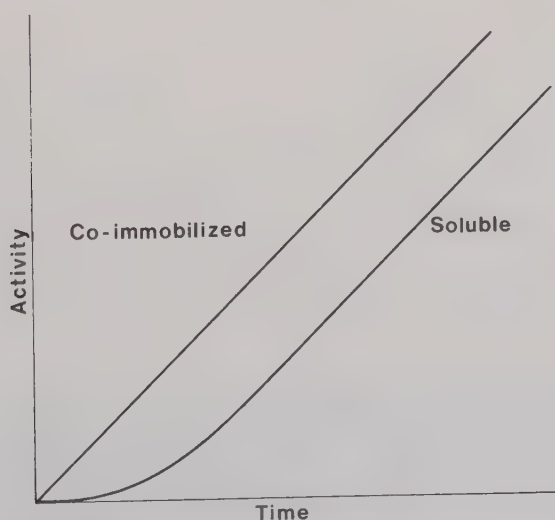
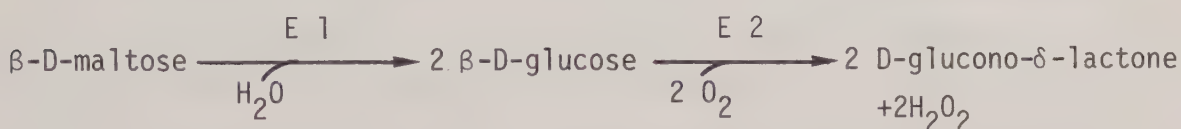


Fig. 6. Schematic representation of the activities of co-immobilized and soluble two-enzyme systems.

3. pH-activity profiles of a co-immobilized two-step enzyme system (II).

The enhanced overall activity in the early stages of a co-immobilized multi-step enzyme reaction can also be shown by comparing the pH optimum for the co-immobilized system to the pH optimum of the same system in solution.

In the model system amyloglucosidase (E 1) and glucose oxidase (E 2) were co-immobilized on CNBr-activated Sepharose.



The two enzymes have pH optima that differ by 1.6 units, and the pH optimum of the overall reaction was, not surprisingly, found to be between the two separate optima. The exact position was dependent on the activity ratio between the enzymes (E 1: E 2). An increased E 1 activity shifts the optimum towards the optimum of E 2, since the overall reaction becomes more dependent on E 2 activity, which is then more rate-limiting. If E 2 is increased, however, the opposite is true.

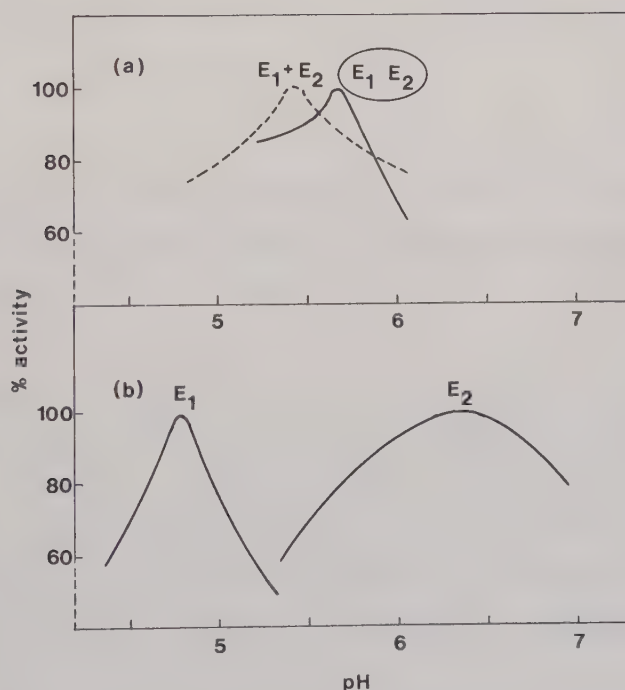


Fig. 7. pH activity profiles for amyloglucosidase (E 1) and glucose oxidase (E 2) or the coupled two-enzyme system (E 1 + E 2) when co-immobilized to Sepharose (E 1:E 2 = 1:1.7) or in free solution (E 1:E 2 = 1:1.5). The optimum activities have been set at 100 %.

The pH optimum of a co-immobilized system with a particular E 1:E 2 was displaced compared to the optimum of the equivalent soluble system, as is seen from Fig. 7. The explanation is that the second enzyme has a higher efficiency in the co-immobilized system than in the free system, during the initial phase of the reaction. This leads to a different effective activity ratio, although the potential activities (as measured at separate optima in excess of substrate) are identical. The higher E 2 efficiency is a result of the enrichment of the intermediate within the microenvironment, and it gives rise to a less E 1-dependent pH optimum, that is the optimum is displaced towards the optimal pH for E 2.

The pH shift was found to vary with the ratio of the potential enzyme activities (Table 2). This reflects the change in E 2 efficiency with different ratios. The E 2 efficiency, which also governs the efficiency of the overall reaction, has been defined as how well the second enzyme in the reaction sequence utilizes the product of the first reaction compared to utilization by an excess amount of soluble E 2 (Hultin, 1974):

$$\% \text{ E 2 efficiency} = \frac{k_{\text{overall}}}{k_{\text{E 1}} (\text{in excess E 2})} \cdot 100$$

For most ratios between the two enzymes, the efficiency is higher for co-immobilized enzymes than for soluble or separately immobilized enzymes (Bouin et al., 1976).

Another way of studying the initial increase in efficiency of E 2 is by measuring the lags or transient times. These have minima at pH optima, and the lags are considerably shorter for the co-immobilized system than for the soluble counterpart. Johansson et al. (1974) found that the difference in lag coincided with the difference in pH optimum between co-immobilized and soluble systems with identical ratios.

TABLE 2. pH optima and E 2 efficiencies (%) for soluble and co-immobilized systems comprised of amyloglucosidase (E 1) and glucose oxidase (E 2) with different ratios between the potential activities (as determined at the separate pH optima for the respective enzyme in excess of substrate). E 1 activity was kept constant.

SOLUBLE ENZYMES			CO-IMMOBILIZED ENZYMES		
E 1:E 2	% E 2 efficiency	pH optimum	E 1:E 2	% E 2 efficiency	pH optimum
1:1.5	15	5.43	1:1.7	38	5.68
1:2.6	19	5.26	1:3.8	40	5.57
1:6.0	23	4.94	1:5.1	46	5.27
1:10	50	4.82	1:7.1	72	4.82

CO-IMMOBILIZED COFACTOR-DEPENDENT ENZYME AND COFACTOR

Cofactors are relatively small, non-protein molecules required by certain enzymes for efficient performance of catalytic function (Mahler and Cordes, 1973). If the cofactors are "self-regenerating", they are needed only in catalytic amounts, as for example the prosthetic groups, which are cofactors covalently bound to the enzyme molecule. Most cofactors, however, must be added in stoichiometric amounts since they are chemically changed after the enzyme reaction and cannot be re-used by the enzyme until they have been regenerated by other means.

Addition of such stoichiometric amounts of the expensive cofactors is impossible for economic reasons. Thus the development of continuous reactors involving immobilized cofactor-dependent enzymes demands repeated use of the cofactors, by retention or recovery, and regeneration, preferably in situ. The co-immobilization of enzyme and cofactor is one approach permitting continuous cofactor re-use (IV).

1. Retention methods for cofactors.

In the most simple case when the cofactor remains unchanged after the catalytic reaction, retention of the cofactor within an enzyme reactor is a useful method of improving the economy of the system and can be accomplished by for example:

- covalent coupling to a particulate support,
- covalent coupling to soluble polymers, or homopolymerization of the cofactor for use in hollow fibres or ultrafiltration reactors,
- covalent coupling to the enzyme surface,
- use of whole cells.

In every case, the main problem is to achieve a system which has good interaction between the cofactor and its proper site on the enzyme. Such interactions were studied for the model system glycogen phosphorylase b and AMP.

2. Immobilized AMP for permanent activation of phosphorylase b (III).

Glycogen phosphorylase b from rabbit muscle has an absolute require-

ment for an effector, AMP, for its catalytic activity. The native AMP can, however, be replaced by AMP-analogues. N^6 -(6-aminohexyl)AMP in free solution caused 85 % activation, but the maximum activation decreased to about 39 % and 20 % respectively on binding the analogue to soluble dextran or Sepharose beads, probably as a result of steric hindrance by the support.

The interaction between free enzyme and Sepharose-bound AMP was so strong that a fraction of the enzyme was "hydrophobically" immobilized until it released completely on washing with ethylene glycol solution. A similarly strong adsorption was used by Shaltiel and Er-El (1973) for purification purposes. The reversible hydrophobic binding of enzyme to Sepharose that had first been substituted with an AMP-analogue is, effectively, sequential immobilization of effector and enzyme. The preparations showed about 10 % specific activity in the absence of free AMP.

The phosphorylase was also bound permanently in the second step if propylamino-chains, which had been bound to the gel simultaneously with the AMP-analogue, were activated with glutaraldehyde. Thus preparations were obtained that showed activity even after ethylene glycol washing (unpublished results).

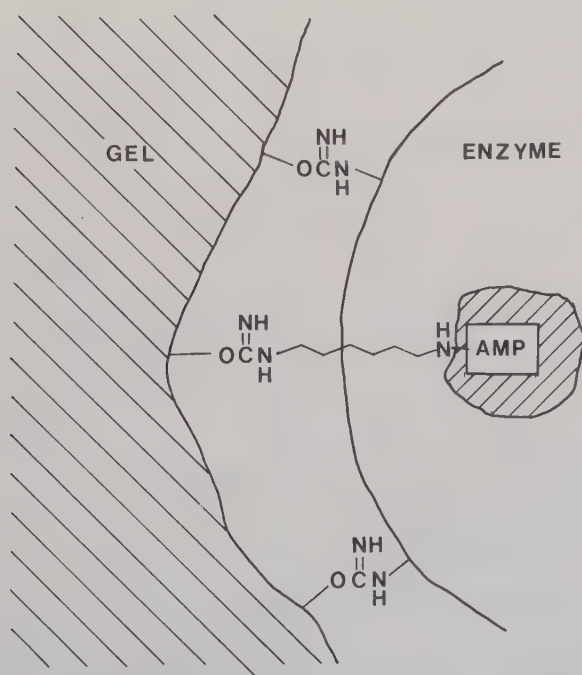


Fig. 8. A schematic drawing of an active phosphorylase b - AMP - Sepharose complex.

Co-immobilization of phosphorylase b and AMP-analogue on Sepharose or glass was a convenient method of obtaining preparations with high internal activity, that is activity due to interactions between immobilized enzyme and immobilized AMP. Fig. 8 depicts schematically the assumed organization (Gestrelus, 1975).

The fact that the co-immobilized preparations could be further activated by addition of free AMP or analogue shows that not all the enzyme subunits are optimally oriented towards the immobilized effector. For a preparation, with 70 μmol AMP-analogue and 42 nmol enzyme subunits per gram dried Sepharose, the internal activity was one third of the maximum activity obtained in saturating concentrations of native AMP, and it corresponds to a specific activity of 10 % (Table 3).

In this context it should be noticed that enzyme immobilization per se was responsible for a decrease in specific activity to about 30 %, although coupling was carried out in the presence of AMP or analogue.

Less efficient activation was obtained with another AMP-analogue, 8- (6-aminohexyl)amino-AMP, both when free and when co-immobilized with phosphorylase b on Sepharose (unpublished results).

TABLE 3. Specific activity of Sepharose-bound glycogen phosphorylase b.

Phosphorylase <u>b</u> preparations	Relative specific activity (%)		
	- free AMP	+ free AMP	+ free analogue (N ⁶ -(6-aminohexyl)AMP)
Native enzyme	0	100 ^{x/}	85
Sepharose-bound enzyme	0	27	26
Sepharose enzyme AMP-analogue	10	30	26
	$\underbrace{\hspace{10em}}$ 1/3 rd internal activity relative to maximum		

^{x/}100 % is equivalent to 14.6 μmol P_i produced per mg protein and minute.

In a different approach a photo-active AMP-analogue (N^6 -6-(2 nitro-4-azido-phenyl)-aminohexyl -AMP) was bound to the phosphorylase molecule itself. The high hydrophobicity of this analogue however, made it very difficult to distinguish between activation due to adsorbed or covalently bound AMP (Larsson and Gestrelus, unpublished results). Hulla and Fasold (1972) made a similar preparation of partially active phosphorylase b with an AMP-analogue covalently bound to the protein via a thioether bond.

To summarize, in all the approaches used, coupling of AMP to a soluble support, to a particulate matrix (sequential or co-immobilization with the enzyme) and to the enzyme molecule itself, the effector could be retained and the interaction between enzyme and AMP was good enough to obtain permanently activated phosphorylase b.

3. Regeneration methods for immobilized cofactors.

During the last few years a number of regeneration methods have been reported that are applicable in immobilized enzyme-cofactor systems:

1. Coupled-enzyme method.
2. Coupled-substrate method.
3. Use of whole cells.
4. Electrochemical regeneration.
5. Chemical regeneration.

The coupled-enzyme method is quite expensive but could become economical if the product of the regenerating enzyme were marketable and if the enzymes were co-entrapped. This method is readily applicable for co-factors bound to soluble polymers (examples and references in Table 6, p. 38), while the regeneration of particle-bound (insoluble) cofactors is more difficult (Larsson and Mosbach, 1974).

The coupled-substrate method can be used for enzymes catalyzing easily reversible reactions and for both soluble and particle-bound cofactors, as is shown in the present study (IV) with co-immobilized alcohol dehydrogenase and NAD-analogue.

Immobilized whole cells contain within the cells a complete enzymic assembly for regeneration, and no addition of coenzyme is necessary as long as suitable substrates are added (Chibata et al., 1974).

Electrochemical regeneration is a fairly new method, which generally involves regeneration of the (soluble) cofactor in an electrolytic cell separated from the reaction column. Its use was recently reported for the reduction of NAD^+ bound to a soluble matrix (Aizawa et al., 1976).

A number of chemical compounds are capable of oxidizing pyridine and flavin cofactors specifically, while reductive agents are few. Chambers et al. (1974) compared chemical regeneration of soluble, polymer-bound NAD^+ by several redox dyes to enzymatic regeneration by diaphorase in a hollow fibre reactor. In the present study (V) flavins have been immobilized for repeated use as regenerators in cofactor-dependent enzyme systems.

4. Co-immobilized alcohol dehydrogenase and NAD(H) analogue (IV).

Horse liver alcohol dehydrogenase and an NADH analogue, N^6 -[[6-amino-hexyl)carbamoylmethyl]-NADH, were co-immobilized on Sepharose. The reduced form of the cofactor with its lower dissociation constant was used to favour binary complex formation between the enzyme and the cofactor (cf. p. 10). In this way a preparation could be obtained with both the NAD(H) analogue and the enzyme covalently bound in such a manner that the coenzyme is fixed in or near the active site of the enzyme (Fig. 9).

Regeneration was accomplished by alcohol dehydrogenase activity using the coupled oxido-reduction between two alternative substrates, ethanol and lactaldehyde. This is a method that is believed to achieve reoxidation of produced NADH without prior dissociation of the cofactor from the enzyme site (the dissociation process is normally rate limiting according to Sund and Theorell, 1963). The reaction was driven towards acetaldehyde formation by removal of this volatile product.

Lactaldehyde could also be replaced with 5 β -dihydrotestosterone, which was converted to the corresponding 3 β -hydroxy steroid if the steroid active isozyme (SS) of alcohol dehydrogenase was co-immo-

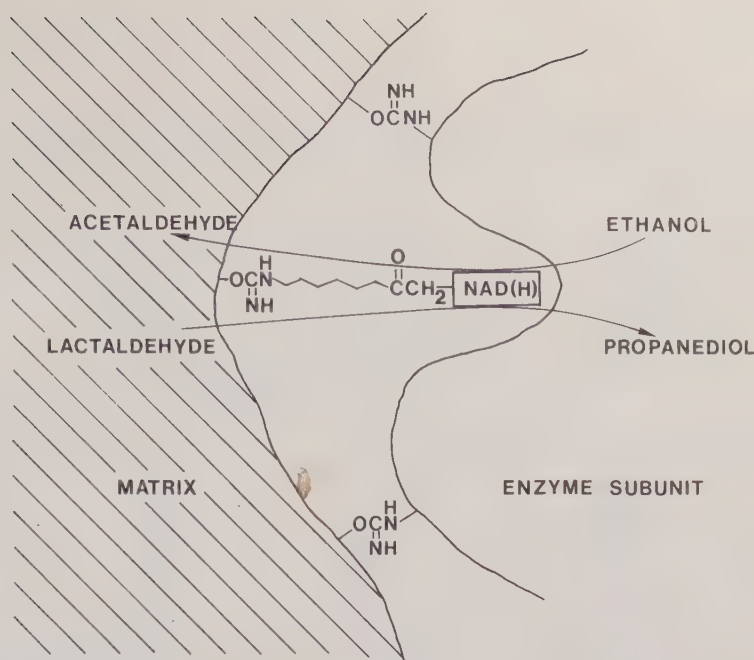


Fig. 9. A schematic drawing of the active alcohol dehydrogenase-NAD(H)-Sephadex complex. The coenzyme is regenerated in situ by the coupled-substrate method, using ethanol and lactaldehyde as substrates. Results show that these immobilized enzyme-coenzyme complexes are responsible for almost all the internal activity of the preparation, that is the activity in the absence of free coenzyme.

bilized with the NADH-analogue. (For preparation of the isozyme see Andersson et al., 1975). (Unpublished observation.)

Several preparations were made with different cofactor/enzyme ratios. As is seen from Table 4, the internal activity, that is the dehydrogenase activity due to interactions with bound cofactor, increased with increasing ratios. For the highest cofactor/enzyme ratio, the internal activity was almost 40 % of the maximum activity obtained in the presence of excess free NAD^+ . A molar ratio of 1/1 was however sufficient to obtain 20 % internal activity, and this surprisingly high activity at a low ratio is ascribed to the immobilization of binary complexes, as has already been discussed (p.10). An approximate statistical calculation according to Green and Toms (1973) of the probability of interaction between molecules randomly coupled on a Sephadex matrix showed that enzymes and cofactors on preparations with such low ratios and low total concentrations should not interact at all since randomly bound molecules are very far apart.

In a confirmatory experiment, a comparable enzyme-cofactor-matrix complex was prepared in the presence of a large excess of native cofactor, that is under conditions not permitting complex formation between enzyme and NADH-analogue, and the negligible internal activity obtained was taken as evidence of the importance of the immobilized binary com-

TABLE 4.

Properties of various alcohol dehydrogenase-NADH-Sepharose preparations.

NAD(H)- analogue/ subunit	Specific internal activity ($\mu\text{mol}/\text{min mg}$) protein	Internal activity relative to maximum (%)	NAD(H) cycles (h^{-1})	Enzyme subunit turn-over (h^{-1})
0.55/1	0.8	18	3400	1700
1/1	0.8	20	1900	1900
10/1	5.7	33	1400	14000
140/1	13.4	40	200	28000

The protein content was between 5 and 15 mg per g dried matrix except for the 0.55/1 preparation which contained about 45 mg/g.

plexes.

The internal activity can also be expressed as cofactor cycling rate. As is seen from Table 4, the highest rate based on the total amount of cofactor was $3400 (\text{h}^{-1})$. Such calculations, however, give values that are much too low for high cofactor/enzyme ratios, since only a small fraction of the cofactor molecules are interacting with enzyme molecules, and a calculation based on the enzyme subunit turnover number (h^{-1}) should give more accurate values in this case. The highest cycling rate thus obtained is of the same magnitude as the maximum rate that has been obtained for NAD(H) in solution with a large excess of soluble enzyme (Schulman et al., 1974).

Attempts were made to use other regeneration methods than the two-substrate method. The coupled-enzyme method, using lactate dehydrogenase, as well as several chemical regeneration methods (with phenazine ethosulphate and 2,6-dichlorophenolindophenol, or potassium ferricyanide) failed however. This was probably due to steric hindrance, the bound cofactor to be recycled being shielded by the alcohol dehydrogenase molecule. Again this supports the concept of active enzyme-cofactor complexes, making the two-substrate regeneration method the most favourable, since it works without dissociation of the cofactor from the enzyme.

5. Continuous regeneration of NAD(P)^+ by immobilized acriflavin (V).

All previously used recycling methods except electrochemical regeneration involve the addition of soluble reactants to the enzyme-cofactor reactor. Electrochemical regeneration still has drawbacks, such as insufficient specificity, and has only rarely been carried out in situ. Various electron acceptors were therefore tested for their ability to fulfill the following requirements for an NAD(P)^+ regenerator:

- specific reoxidation of NAD(P)H to NAD(P)^+
- retention within the reactor
- continuous use, that is the regenerator itself must be regenerated in situ
- non-contaminating
- inexpensive

This specificity limited the choice to phenazine compounds, pyridinium salts, flavines and a few others. They are all fairly cheap, especially those that can be reoxidized by molecular oxygen and therefore are only needed in catalytic amounts. Retention requires that the low-molecular-weight reagents are immobilized via functional groups. Such binding must also be stable to avoid leakage.

When acriflavin, a commercially available flavin analogue, was immobilized on epoxy-activated Sepharose it fulfilled the above requirements quite well. The two amino-groups offer good possibilities for binding, and the linkage was shown to be extremely stable compared to most other bonds. The oxidizing capacity towards NAD(P)H was about 25 % for the immobilized acriflavin compared to the soluble, and the regenerator could be used repeatedly since the reduced acriflavin was immediately reoxidized in aerobic solutions.

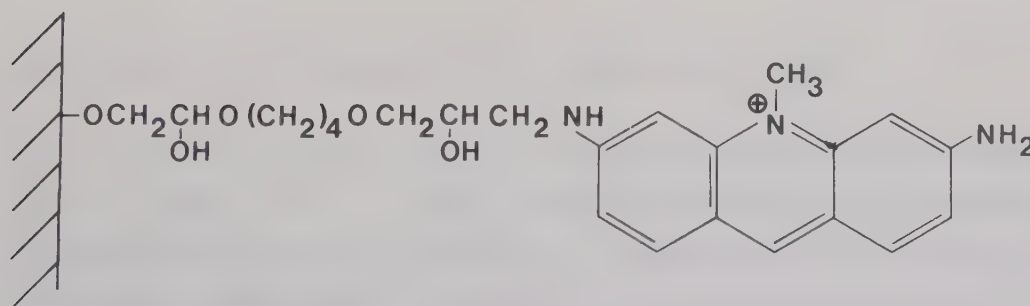
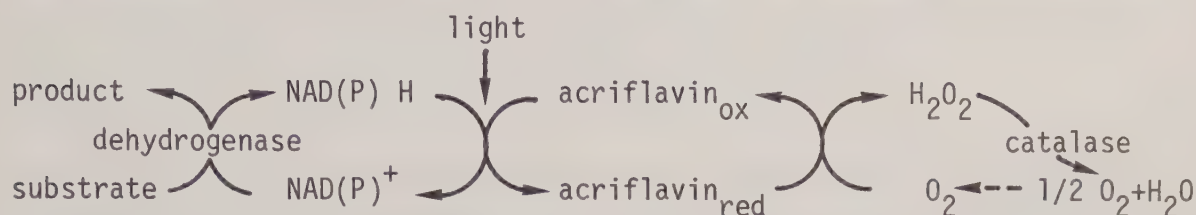


Fig. 10. Acriflavin bound to epoxy-activated Sepharose.

The immobilized acriflavin could be used in a number of different NAD^+ - and NADP^+ -dependent dehydrogenase systems, including lactate dehydrogenase, alcohol dehydrogenase, malate dehydrogenase and glutamate dehydrogenase, leading to recycling of NAD(P)(H) as is seen in the scheme:



One draw-back was the need for light-activation of acriflavin prior to reaction with reduced cofactor, but a strong day-light lamp can easily be built into the enzyme reactor. Another and more serious problem was the contamination by hydrogen peroxide which was formed on reoxidation. This was overcome by using separately immobilized enzyme and acriflavin during long-term operations and by adding catalase or manganese oxide powder (Duvnjak and Lilly, 1976), for enzymic or chemical destruction of the peroxide.

In a long-term experiment, the activity of Sepharose-bound lactate dehydrogenase had declined to zero after more than three days at room temperature. All NAD(H) (100 nmol) had then been reused at least 40 times and the bound acriflavin (770 nmol) about five times, and 225 times more pyruvate had been produced than in a system without cofactor regeneration.

A very low concentration of NAD^+ and a large excess of acriflavin leads, of course, to the most efficient utilization of the cofactor. However, since pyruvate production rate is increased with increasing concentration of NAD^+ , the optimization of the system must depend eventually on the cost of the cofactor and the value of the product.

6. Co-immobilized enzyme-cofactor-regenerator systems.

When immobilized acriflavin (V) or another polymerized regenerating agent (Chambers et al., 1974) is co-entrapped with a dehydrogenase and a polymer-bound (soluble) NAD(H) , an enzyme-cofactor-regenerator system is created that is analogous to the co-entrapped coupled enzymes and polymer-bound cofactor (Davies and Mosbach, 1974, Marconi et al., 1975/76). These systems, and the co-immobilized enzyme-cofactor system

with two-substrate regeneration (IV), all carry out the desired reaction and continuous regeneration of the cofactor within one reactor. Only addition of oxygen and light or regeneration substrates is needed. Other approaches for obtaining such "complete" catalyst systems have also been tried, for example by integrating the regenerating agent in the matrix material (described below, unpublished).

An active enzyme-cofactor-regenerator matrix complex can be prepared by adsorbing a poly-acriflavin layer to glass beads and coupling of lactate dehydrogenase and NAD-analogue, $(N^6-[(6\text{-aminohexyl})\text{carbamoylmethyl}]-NAD)$, to reactive diazo-groups on the acriflavin residues. The immobilization method is related to the use of Bismarck brown for coupling of enzymes (Gray et al., 1974), but the polymer matrix is also a regenerating agent for the cofactor. The activity of the system is reasonably good if the preparation is used in the presence of soluble catalase, or if catalase is also co-immobilized, to destroy the deleterious hydrogen peroxide produced during reoxidation of acriflavin. A major problem is to prevent leakage of cofactor and regenerator from the matrix. A lot of free acriflavin and NAD-analogue seem to be hydrophobically adsorbed on bound molecules, and it is very difficult to remove these without destroying the whole preparation. The activity of the system was, however, unchanged from one long-term run to another.

DISCUSSION

1. Co-immobilization versus immobilization in separate matrices

The following factors are, among others, important for a proper choice between co-immobilization (resulting in dual catalysts) and immobilization in separate matrices (resulting in mixed catalysts):

- enzyme kinetics
- enzyme and cofactor stability
- process conditions.

In Table 5 some examples of advantages of use of dual catalysts and mixed catalysts are collected.

Co-immobilization is beneficial for most multi-step enzyme systems, since it permits exploitation of the fast build-up of a high concentration of intermediates in the microenvironment (Mosbach and Mattiasson, 1976). The gain in overall activity is most extreme if one enzyme is inhibited by the first substrate (Hervagault et al., 1975), while separate immobilization is most suitable if the multi-step enzyme system is feed-back inhibited by a product (Lecoq et al., 1975). Also co-immobilization of an enzyme-system, catalyzing a reaction where one step (not the last) is thermodynamically unfavourable, will create a pull of the equilibrium and give a much higher overall reaction rate (Srere et al., 1973) not only in the initial phase but continuously.

The proximity in co-immobilized systems is also favourable for enzymes that act alternately on a substrate or a cofactor. Examples of this are the degradation of proteins, or starch (Mårtensson, 1974) and the two-enzyme regeneration of NAD^+ (Srere et al., 1973, Wykes et al., 1975). Co-immobilization of a dehydrogenase and the "enzyme-replacing" regenerating agent for NAD^+ , acriflavin, is however only advantageous if catalase is also included in the system, to remove the deleterious hydrogen peroxide which is produced on reoxidation of the reduced acriflavin by oxygen (V).

Positive and negative effects can also be obtained if two metabolically unrelated enzymes are co-immobilized. One example is the regulation by proton-producing (or -removing) enzymes that is shown in the present study (I).

TABLE 5

Some advantages of dual catalysts and mixed catalysts

DUAL CATALYSTS	MIXED CATALYSTS
1. Higher activity in the initial phase of a multi-step enzyme reaction.	1. When products have feed-back inhibitory effects in a reaction sequence or on an enzyme system.
2. Higher overall rate in systems containing thermodynamically unfavourable steps.	2. Less sensitive to creation of specific microenvironments (such as changed pH).
3. When enzymes act alternately upon a substrate or a cofactor.	3. Easy to replace one enzyme if it has much lower stability than the others in the system.
4. When products have feed-back activating effects.	4. Easier to vary the activity ratio between the participating enzymes.
5. For efficient interaction between immobilized cofactor-dependent enzyme and its cofactor.	5. When coupling must take place under very different conditions.
6. When protein-protein or protein-cofactor (effector) interactions will stabilize an enzyme.	6. When the separate enzyme catalyzed reactions require very different conditions(might be created by use of different matrices).
7. When a multi-step enzyme reaction must be carried out under unfavourable conditions (for example low substrate conc., extreme pH-values).	

Moreover, the proximity obtained on co-immobilization of a cofactor-dependent enzyme and its cofactor is very beneficial for high activity (IV), especially since the molecules could be bound as binary complexes to the matrix. Separately immobilized enzymes and cofactors would only have infinitesimal possibilities for interaction, due to steric hindrance by (particulate) matrices (Wykes et al., 1975).

Enzyme stability is a crucial problem for all applications of enzymes. Many matrix materials are quite expensive and the operational stability of the enzymes and the matrix must be good enough to make immobilization or co-immobilization economically feasible. Good heat resistance is desirable since high operation temperatures reduce the growth of microorganisms that can destroy the enzymes as well as the matrix materials (Cf. p. 7).

An apparent stabilization on immobilization of large concentrations of enzymes has been noticed for many enzymes. This effect is thought to arise in strongly diffusion limited systems, where an activity loss in the outer part of an enzyme-matrix only results in substrate permeation into regions containing active molecules, which were previously cut off from contact with substrate since all substrate had been converted at the surface (Ollis, 1972)

However, co-immobilization seems to have a real stabilizing effect due to protein-protein interactions. Inert proteins like serum albumin and haemoglobin are thus very often included during immobilization of enzymes (Broun et al., 1968, Dahlquist et al., 1973, Campbell and Chang, 1975). Immobilization, and even co-immobilization, can also stabilize by steric hindrance, leading to the avoidance of unfavourable interactions. One example is the increased protein stability in the presence of a co-immobilized protease compared with a system with a free protease (I). Another example is reported by Mattiasson et al. (1974b) concerning phosphofructokinase and aldolase. It is thought, that the interaction between these enzymes involves hydrolysis by aldolase of some fructose-1,6-diphosphate molecules bound to the phosphofructokinase and thereby deactivating the latter. The reduced influence of high aldolase concentrations when the enzymes are co-immobilized or immobilized in separate matrices is consequently ascribed to steric hindrance.

If one enzyme activity in a multi-enzyme system is much less stable than the others, then immobilization on separate matrices is to be preferred, thus making it easier to add the missing enzyme activity. It might even be possible to exchange inactivated enzyme for a fresh preparation if one kind of enzyme matrix can be separated from another. This could be accomplished for example by immobilizing one enzyme on a magnetic support (Gellf and Boudrant, 1974).

For co-immobilized enzyme-cofactor systems the specific activities of the enzymes were found to be markedly higher than those of enzymes that had been immobilized alone (III, IV). The enzymes in position for interaction with co-immobilized cofactor also had a much higher stability towards heat and long-term storage than an uncomplexed enzyme (IV). Co-immobilization of enzyme and cofactor is of course of little value unless the immobilized cofactor has at least the same operational stability as the immobilized enzyme (Wykes et al., 1975).

Co-immobilized enzymes and cofactors will probably be preferred in analytical devices as well as in future medical applications such as enzyme replacement therapy and artificial organs, where compact and very efficient systems are needed.

In large scale reactors, the use of mixed catalysts makes it easier to control enzyme ratios and activities. Other advantages are that coupling can take place under optimal conditions for each enzyme, and that the reactions may be carried out under different conditions, provided the enzyme matrices are used in separate reactors or the matrices create different microenvironments.

However, if a multi-step reaction must be carried out under unfavourable conditions, with rate-limiting substrate concentration or extreme pH-values of the surrounding medium, then co-immobilization, with its higher efficiency for the second and following enzymes, will be the method of choice (II), and since co-immobilized multi-step enzymes are more efficient during the initial phase, their use in reactors will also permit higher flow rates.

Continuous reactors involving cofactor-dependent enzymes demand repeated use of the expensive cofactors, as was discussed on p. 20. Co-immobilized enzyme-cofactor systems have high efficiency, acceptable stability (at room temperature), the main disadvantage being the limitations on regeneration methods.

Two-enzyme regeneration of a cofactor when all three molecules are immobilized seems to be almost impossible (unpublished results) and two-substrate regeneration which was successfully used for alcohol dehydrogenase (IV) is only applicable for readily reversible enzyme reactions. Thus, the development of a highly specific electrochemical method or an in situ chemical regenerating agent, maybe by creating a "bis-cofactor" that is "self-regenerating", is an important objective.

2. Co-immobilized enzymes and cofactors as biological model systems

Compartmentalization is now being recognized as a necessary structural feature of all living cells and cell systems. In a review by Srere and Mosbach (1974), the different levels of compartmentalization were discussed, including the "unseen compartments", the microenvironments. Since most enzymes are believed to exist as membranebound or solid-state assemblies, or in gel-like surroundings, such individual microenvironments are bound to exist. They can be created either by the chemical nature of the membrane or assembly or by the impeded diffusion near a surface or in a region of increased viscosity. One example of a microenvironmental effect is the change in pH optimum after solubilization of a membrane-bound acetylcholine esterase (Silman and Karlin, 1967).

It is, however, very difficult to distinguish the microenvironments in vivo and to evaluate their importance. When using enzymes bound to artificial polymer particles as model systems, one microenvironmental effect at a time can be studied, and since the effects can very often be magnified this means that they are more easily detected with standard instruments.

Two important consequences of the microenvironment in vivo seem to be:

- Compartmentalization of intermediates, thereby minimizing competition with other metabolic pathways and protecting unstable intermediates.
- Reduced transient times (no lags), permitting rapid triggering of overall enzyme activity.

In a very recent report, Bryce et al. (1976) presented kinetic results from a natural two-enzyme system, aspartate aminotransferase and malate dehydrogenase; enzymes that have been shown to constitute a complex (Backman and Johansson, 1976). The overall activity of the system showed no lag phase, and there was no equilibration between the produced intermediate (oxaloacetate) and bulk oxaloacetate, facts that indicate some kind of compartmentalization of the intermediate. Welch and Gaertner (1975) reported for Neurospora crassa that the aromatic complex had lags that were 10-15 times shorter than would be expected for a hypothetical unaggregated system.

Similar effects have previously been found for co-immobilized model systems. Mattiasson and Mosbach (1971) showed the absence of lag for a co-immobilized three-enzyme system, and the distorted pH profiles obtained for enzymes co-immobilized with a proton-producing (or re-moving) enzyme in spite of a constant bulk pH (I) is an example of slow equilibration of a product with the surrounding medium.

In enzyme reactions carried out by for example kinases, phosphatases dehydrogenases, and in pathways like glycolysis and Krebs cycle, protons are produced or removed, and can affect enzymes in their neighbourhood through their accumulation in the microenvironment. In such cases, local proton concentrations may regulate the activity of some of the participating enzymes. In the present model study (I) it was shown that such possible pH regulation is dependent on the buffer capacity of the system. Very little is known, however, about buffer capacities in cells, but it is known that local pH values and gradients exist and that protons are general effectors of enzyme activity. A simple example is the lysosomal pH necessary for, and produced by, lysosomal enzyme activity (Reijngoud and Tager, 1973). Other ions, for example inorganic phosphate (Kravitz and Guarino, 1958) may also have comparable regulative function.

Apart from the microenvironmental effects, the proximity is supposed to be important for multi-enzyme systems in vivo. Srere et al. (1973) studied an immobilized three-enzyme system with one thermodynamically unfavourable step, to resolve the apparent discrepancy between the known rate of the citric acid cycle and the low apparent concentration of free oxaloacetate in mitochondria. Srere (1972) had postulated an

organization of the Krebs cycle enzymes in the mitochondrial matrix, and the co-immobilized model-system showed a 400 % enhanced activity compared to a corresponding free system, a fact that was ascribed to the close proximity of the participating enzymes, permitting continuous removal of produced oxaloacetate.

Cofactors are also compartmentalized in vivo. In mitochondria for example one fraction of NAD(P) is retained in an environment of tightly membrane-bound dehydrogenases (Mahler and Cordes, 1973). Restricted diffusion of soluble cofactor has also been shown to occur in immobilized enzyme systems (Wykes et al., 1975, Fink et al., 1975). Moreover, complete retention could be imposed by covalent binding of NAD(H), either by co-immobilization with an enzyme to a matrix in the form of a binary complex (IV) or by binding directly to the enzyme (Venn et al., 1976), thereby mimicking a prosthetic group.

Flavins are very often native prosthetic groups, being covalently bound to enzyme molecules in vivo, and for a flavoenzyme such as lipoyldehydrogenase the reduction of NAD is postulated to take place when the flavin as well as the nicotinamide cofactor are bound to the enzyme (Mahler and Cordes, 1973). Such hydrogen transfers have recently been studied in model systems with cofactor fragments covalently bound to each other (Blankenhorn, 1975).

However, although model studies on immobilized enzymes and likewise immobilized cofactors might give valuable biological information, all enzyme-cofactor-matrix preparations have so far been used for purely practical and economic reasons.

3. Technical and medical applications of co-immobilized enzymes and cofactors.

Systems of co-immobilized enzymes and cofactors are not yet in commercial use, the main obstacles being enzyme and cofactor instability and cofactor regeneration problems (cf. the discussion on pp 32-34). Several enzyme systems have however been suggested for applications in industrial processes, for analytical instruments and in medical therapy. Some of these that have already been tested on the laboratory scale are listed in Table 6. The multi-enzyme systems seem to be concentrated on food processing, process regulation and clinical analysis, that is in fields where the proximity and microenvironmental effects can be exploited to give high total

conversion of a substrate, or a rapid response and high sensitivity towards low substrate concentrations. On the other hand, most of the cofactor-dependent systems are obviously model systems used to develop cofactor regenerating methods. More complicated multi-step enzyme systems involving cofactor-dependent enzymes will however find use, for example in the pharmaceutical industry, once they have been developed. Well-known examples are systems carrying out steroid conversions. Although the problems may seem large and alternative approaches using enzyme analogue catalysts, so called synzymes, have been suggested, there is no doubt that enzyme technology using immobilized and co-immobilized enzymes and cofactors will be further exploited commercially. To quote Skinner (1975): "Any process that can benefit from catalysis potentially can benefit from enzymes".

TABLE 6. Some suggested practical applications for co-immobilized enzymes and cofactors.

ENZYME/COFACTOR SYSTEM	APPLICATION	REFERENCE
α -amylase+pollulanase	conversion of starch	Mårtensson (1974)
glucose oxidase+catalase	1. desugaring of egg products	Hultin (1974)
	2. production of gluconic acid	"_
	3. determination of glucose	Mattiasson et al. (1976)
asparaginase+glutaminase	cancer therapy	Fernandes et al. (1975)
invertase+glucose oxidase	determination of sucrose	Inman and Hornby (1974)
tryptophanase+lactate dehydrogenase	determination of tryptophan	Ikeda and Fukui (1974)
asparate aminotransferase+maltate dehydrogenase	determination of aspartate	Ikeda et al. (1974)
β -galactosidase	determination of lactose	Cordonnier et al. (1975)
maltase	"_ maltose	"_
invertase	"_ sucrose	"_
alcohol dehydrogenase+dextran-NAD + poly-FMN (co-entrapped)	production of acetaldehyde	Chambers et al. (1974)
alcohol dehydrogenase+aldehyde dehydrogenase+diaphorase (+ free NAD)	production of acetic acid	Chambers et al. (1974)
lactate dehydrogenase+glutamate dehydrogenase+dextran-NAD (co-entrapped)	determination of glutamate	Davies and Mosbach (1974)
lactate dehydrogenase+alanine dehydrogenase+polyethyleneimine-NAD (co-entrapped)	determination of alanine	Marconi et al. (1975/76)
alcohol dehydrogenase+NADH oxidase(+ free NAD)	production of acetaldehyde	Kawai (1975)
alcohol dehydrogenase+lactate dehydrogenase+formyl PEI-succinyl-NAD (co-entrapped)	production of acetaldehyde and lactate	Wykes et al. (1975)
alcohol dehydrogenase+NADH (both Sepharose-bound)	production of acetaldehyde + propanediol alt. 3- β -hydroxy steroids	IV

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